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## Lipid Binding to the Amphipathic Membrane Protein Cytochrome $b_5$ (electron spin resonance/lipid spin labels/endoplasmic reticulum membrane)

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**ABSTRACT** The lipid binding properties of the membrane protein cytochrome  $b_5$  (detergent-extracted from calf liver microsomal preparations) were characterized by studying the interaction of spin-labeled lipids (5-, 12-, and 16-doxyloleic acid and 5- and 16-doxyloleophosphatidylcholine, where doxyl refers to the nitroxide moiety) with cytochrome  $b_5$ , using electron spin resonance spectroscopy. The intact cytochrome  $b_5$  molecule immobilizes all of the lipid spin labels, while the segment of cytochrome  $b_5$  released by trypsin does not affect lipid mobility. The immobilization of lipid spin labels on the hydrophobic surface of intact cytochrome  $b_5$  is not appreciably altered by associating the protein with liposomes. Differences in polarity of the lipid binding sites between cytochrome  $b_5$  and phospholipid vesicles were also observed. The lipid binding sites on cytochrome  $b_5$  are hydrophobic by conventional criteria, but are more polar than the interior of fluid phospholipid bilayers.

Sufficient evidence is available to indicate that some membrane proteins are at least partially buried in the lipid bilayer continuum of the membrane. The intimate contact between these hydrophobic polypeptide regions of the membrane protein and surrounding lipids is a feature common to most of the current ideas concerning membrane structure. There must be, then, an interfacial region between the amino acid side groups and lipid acyl chains, and the molecular properties of the lipid in this interfacial region may be very different from those of the lipid in the adjacent bilayer (1).

Recent studies of lipid-protein interactions in model membranes formed from cytochrome oxidase and its associated phospholipids provide considerable evidence for a layer of lipid (boundary lipid) that is highly immobilized (1), presumably by the hydrophobic surface(s) of the protein. The cytochrome oxidase complex is composed of six or seven polypeptide chains (2) whose structural relationship in the complex is poorly understood. Our purpose in the present study is to examine the hydrophobic protein surface buried in the membrane when the functional protein consists of single polypeptide chain. We have selected a well-characterized amphipathic membrane protein, cytochrome  $b_5$  from calf liver endoplasmic reticulum. This protein consists of a single polypeptide chain and has been isolated both with and without the hydrophobic segment thought to serve as the region of attachment to the membrane (3). When isolated with detergents, the intact cytochrome  $b_5$  can be freed of phospholipid and detergent contaminants and can, under appropriate con-

ditions, be reassociated with the membrane with its catalytic properties apparently unaltered by the prior extraction procedures (4). The hydrophilic portion of the cytochrome  $b_5$  molecule, containing approximately 65% of the amino acid residues, can be cleaved from the membrane by hydrolytic enzymes, using either trypsin (5) or pancreatic lipase (6). After purification this yields a heme-containing cytochrome  $b_5$  fragment lacking the hydrophobic tail assumed to serve for membrane attachment. Thus, the lipid binding properties of this well-characterized membrane protein can be examined when the presumptive hydrophobic binding region is present (detergent-extracted cytochrome  $b_5$ ) or absent (trypsin-extracted cytochrome  $b_5$ ). Using electron spin resonance we have examined the behavior of lipid spin labels binding to cytochrome  $b_5$  in order to approach the following questions: (i) Is lipid binding detectable, and, if so, is such binding confined to one region of this membrane protein? (ii) How does the mobility of the lipid at the hydrophobic protein surface differ from that seen in the bilayer regions, and is any difference maintained in the presence of contiguous bilayer regions? (iii) How does the polarity of the binding surface compare with that of the interior of the bilayer? We attempt to answer these questions by examining the behavior of the lipid spin labels shown in Fig. 1 as they interact under various experimental conditions with the cytochrome  $b_5$ .

### MATERIALS AND METHODS

All reagents were the highest commercially available grade and were used without further purification. Trypsin (Mann), pepsin, and horse heart cytochrome  $c$ , type VI (Sigma), and the fatty acid spin labels (Syva) were used. The phospholipid spin labels, 5- and 16-doxyloleophosphatidylcholine, were the gift of T. Marriott and T. Micka, and were prepared and characterized by standard literature procedures (7). Protein determinations were performed by the method of Lowry *et al.* (8), phosphate was measured by the procedure of Fiske and Subbarow (9), and acrylamide disc gel electrophoresis methods were similar to those of Weber and Osborn (10). Lipids were extracted from washed calf liver microsomal fraction by the method of Folch *et al.* (11) and were stored in chloroform under nitrogen at  $-20^\circ$ . Electron spin resonance (ESR) spectra were recorded on a Varian E-3 9.5 GHz spectrometer using a Varian 620/i 8K computer to digitize and integrate the data (12).

*Preparation of the Cytochromes  $b_5$ .* All procedures were performed at  $4-6^\circ$  in the cold room. Detergent-extracted cytochrome  $b_5$  was prepared from calf liver microsomes following the procedure that Spatz and Strittmatter (3) have reported

Abbreviations: ESR, electron spin resonance; doxyl, the 4',4'-dimethyloxazolidine-N-oxyl derivative of the corresponding keto precursor (5- and 16-doxyloleophosphatidylcholine refer to the corresponding doxyloleic acids acylated to lysolecithin).



for detergent extraction of cytochrome  $b_5$  from rabbit liver microsomes. Briefly, the procedure involves lipid extraction of extensively washed microsomal suspensions with cold aqueous acetone, followed by stirring overnight in 1.5% Triton X-100 at 4°. The supernatant obtained after centrifugation was fractionated on a DEAE-cellulose column, and further purified by gel filtration on Sephadex G-100 in the presence of deoxycholate. The protein fraction was freed from detergents by passage through a Sephadex G-25 column that had been equilibrated with 0.1 M Tris-acetate buffer, pH 8.1. In aqueous solution in the absence of detergents this cytochrome  $b_5$  aggregates as an octomer with an apparent molecular weight of about 120,000 (3).

Trypsin-extracted cytochrome  $b_5$  was isolated from calf liver microsomes by a modification of the procedure described by Omura *et al.* (5). Liver microsomes were extensively washed and incubated overnight at 4° in 0.05 M potassium phosphate buffer, pH 7.5, containing 15 mg of trypsin per 100 mg of microsomal proteins. The trypsin-extracted proteins were applied to a DEAE-cellulose column equilibrated with 0.05 M potassium phosphate buffer, pH 7.5, and the heme protein was eluted with 0.18 M KCl in this buffer and further purified by passage through Sephadex G-100. The proteins from each preparative procedure were concentrated to 3 mg/ml and stored in 0.02 M Tris-acetate buffer, pH 8.1, at -20°.

**Preparation of Spin-Labeled Samples.** For binding of the doxylstearic acid probes (I-III, Fig. 1) to the two cytochrome  $b_5$  species, the protein in 0.02 M Tris-acetate buffer, pH 8.1, was added to a tube containing a dried film of the spin label, so that the molar ratio of protein:spin label was 8:1 (assuming molecular weights of 11,000 and 16,700 for the trypsin-extracted and detergent-extracted cytochromes  $b_5$ , respectively). In one set of experiments the buffer used was 0.1 M potassium phosphate buffer, pH 6.5. The buffer used had no effect on the ESR spectrum. The detergent-extracted cytochrome  $b_5$  was labeled with the phospholipid spin labels (IV, V, Fig. 1) in the same manner, but followed by low power sonication for 5 min while cooling the sample in an ice bath. This sample was then diluted with sucrose in 0.1 M Tris-acetate buffer, pH 8.1, to give a final concentration of 10% sucrose. The spin labeled protein was concentrated and separated from free spin label vesicles by centrifuging at  $100,000 \times g$  for 5 hr. Initially the protein:spin label ratio was 8:1, but a small portion of the spin label is recovered in the float after centrifugation, so that the actual amount of spin label was somewhat less.

Aqueous dispersions of spin-labeled microsomal lipids were prepared by adding 0.1 M Tris-acetate buffer or 0.1 M potassium phosphate buffer to a dry film of microsomal lipids (12  $\mu$ moles of phospholipid, 0.12  $\mu$ mole of lipid spin label) and the mixture was sonicated for 5 min with cooling. Microsomal lipids contain on the order of 15-20% neutral lipids (cholesterol and triglycerides) (13), so the actual spin label:total lipid ratio is somewhat lower than the calculated 100:1 phospholipid:spin label ratio would indicate. The membranes of the microsomal fraction were labeled by adding the aqueous membrane suspension to a vial containing a dry film of the doxylstearic acid (I-III), with 5  $\mu$ g of spin label/mg of membrane protein, and sonicating for 2 min on ice.

For reconstitution of detergent-extracted cytochrome  $b_5$  with microsomal lipids, the procedure was adapted from

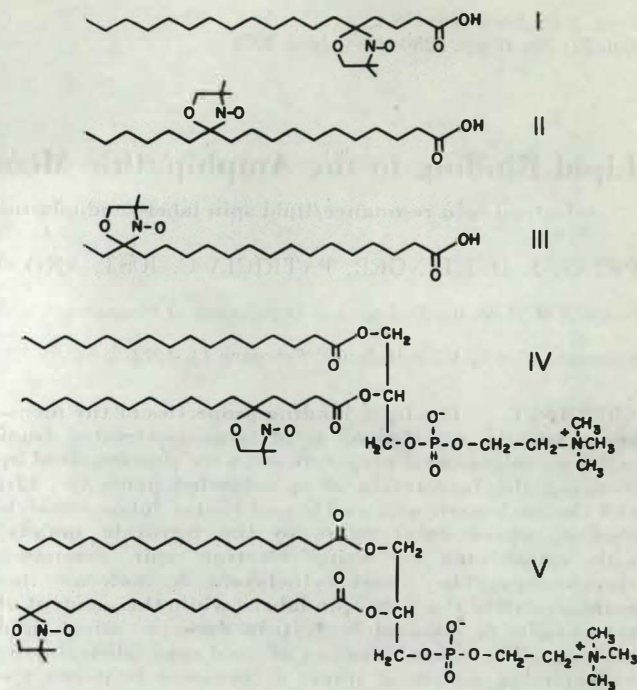


FIG. 1. Lipid spin labels. I-III, 5-doxylstearic acid, 12-doxylstearic acid, and 16-doxylstearic acid; IV and V, 5-doxylphosphatidylcholine and 16-doxylphosphatidylcholine. (Doxyl refers to the 4',4'-dimethylloxazolidine-N-oxyl group.)

Strittmatter *et al.* (4). The protein in 0.1 M Tris-acetate buffer, pH 8.1, was added to a dry film of microsomal lipids and sonicated at low power for 2 min on ice, followed by mixing for 30 min at 37° under nitrogen. The sample was pelleted in 10% sucrose by centrifuging at  $105,000 \times g$  for 4 hr. The pellet was assayed for protein and phosphorus and labeled with 16-doxylstearic acid (III) by bath sonicating the lipid-protein complexes in buffer with a dry film of the spin label at 37° for 2 min. The labeling was kept constant at a molar ratio of protein:spin label of 8:1. Samples used in the ESR experiments were divided into four aliquots for characterization by (1) electron microscopy, using negative staining with 1% sodium phosphotungstate, pH 7, (2) extraction with chloroform:methanol and determination of lipid-extractable phosphorus, (3) protein determination, and (4) application to a continuous sucrose gradient (0-42%), centrifugation at  $250,000 \times g$  for 12 hr, and monitoring the fractions spectrophotometrically at 280 nm.

## RESULTS AND DISCUSSION

**Purity and Molecular Weights of the Cytochromes  $b_5$ .** The protein isolation procedures outlined above result in the isolation of two heme-containing proteins, one released by detergent, and one released by trypsin. Each protein migrated as a single molecular weight species when subjected to sodium dodecyl sulfate gel electrophoresis. Judging by the migration rate relative to marker proteins (10), as shown in Fig. 2, the molecular weight of detergent-extracted cytochrome  $b_5$  was calculated to be about 16,000. The gel on the right in Fig. 2 shows the difference in migration distances between the two cytochrome  $b_5$  species, and corresponds to a difference in molecular weights of approximately 4000. These molecular



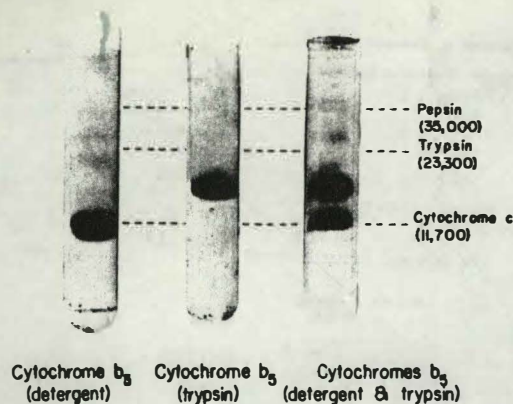


FIG. 2. Sodium dodecyl sulfate acrylamide disc gel electrophoresis of the two cytochrome  $b_5$  preparations. Protein fractions were solubilized by heating in 0.1% sodium dodecyl sulfate and 50  $\mu$ g of each fraction were applied to 15% cross-linked gels. Electrophoresis was performed in 0.05 M sodium phosphate buffer (pH 7.5) containing 0.1% sodium dodecyl sulfate (10), and gels were stained in 1% Fast Green (Eastman) in 10% acetic acid. The broken lines represent the migration distances of three marker proteins.

weights are in general agreement with those reported for the enzyme and detergent-extracted cytochromes  $b_5$  isolated from the livers of several mammalian species (3, 5, 6, 14).

*The Cytochromes  $b_5$  Show Different Lipid Binding Properties.* Fig. 3 shows the results of combining the doxylstearic acids in solution with the two cytochrome  $b_5$  species, detergent-extracted and trypsin-extracted. Each of the three isomers of doxylstearic acid is markedly immobilized by detergent-extracted cytochrome  $b_5$ . In marked contrast, each of these isomers exhibits rapid isotropic tumbling when the protein present is trypsin-extracted cytochrome  $b_5$ , although the motion is reduced slightly due to the viscosity of the solution. There is no doubt that the fatty acid spin labels bind to the intact cytochrome  $b_5$  and do not appreciably bind to trypsin-extracted cytochrome  $b_5$ .

The detergent-extracted cytochrome  $b_5$  preparation used in this experiment was obtained by treating microsomes with the detergent Triton X-100, and subsequently purifying the protein in the presence of deoxycholate. This raises the question of whether the interaction of the lipid probes with the cytochrome  $b_5$  preparation involves interaction with lipid and/or detergent contaminants bound to the protein rather than direct interaction with the protein surface. Two lines of evidence argue against this possibility. First, the procedure described by Spatz and Strittmatter (3) for the detergent extraction of cytochrome  $b_5$  from rabbit liver microsomes, which we followed rigorously, yielded a protein fraction that contained no detectable amounts of extractable lipids or deoxycholate and no detectable lipid-extractable phosphorus. The comparable cytochrome  $b_5$  preparation used in this study was found to contain much less than 1 mole of phosphorus per mole of protein. Second, we repeated the experiment after freeing the cytochrome  $b_5$  of possible detergent and lipid contaminants by the wash procedure utilized by Ito and Sato (15) for delipidating detergent-extracted cytochrome  $b_5$ . The protein was washed three times with cold 90% acetone, resolubilized in 4.5 M urea, and dialyzed extensively against a urea-free buffer. The ESR spectra of the doxylstearate spin labels in association with the acetone-washed protein were es-

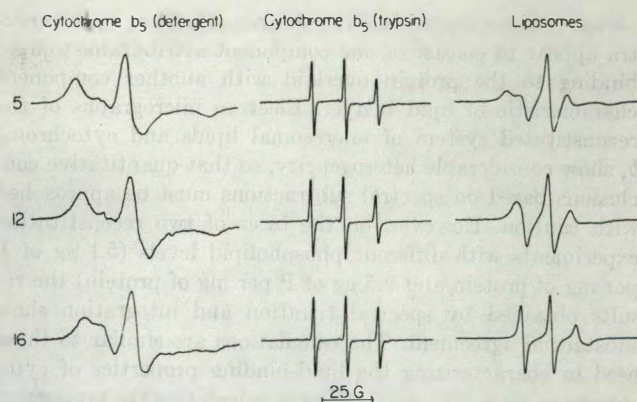


FIG. 3. ESR spectra of 5-, 12-, and 16-doxylstearic acids (from top to bottom) in solution with detergent-isolated cytochrome  $b_5$ , trypsin-extracted cytochrome  $b_5$ , and aqueous dispersions of microsomal lipids. The samples were at room temperature and the spectra have been normalized to the same vertical scale.

entially identical to those seen at the left in Fig. 3. Clearly, trypsin-extracted cytochrome  $b_5$ , which lacks the hydrophobic tail, has no detectable effect on the spin labels moving freely in solution, whereas detergent-extracted cytochrome  $b_5$  causes strong immobilization of the spin labels. Therefore, we conclude that the hydrophobic peptide segment of native cytochrome  $b_5$  is responsible for the immobilization of the stearic acid spin labels.

To appreciate the degree of immobilization of lipid spin labels bound to the hydrophobic segment of cytochrome  $b_5$ , it is useful to examine the motion of the same spin labels in lipid bilayers with no hydrophobic protein present. Using lipid spin labels, it has been established independently in vesicles (7) and hydrated multilayers (16) that motion increases along the fatty acid chains in lipid bilayers, culminating in marked fluidity at the center of the bilayer. This behavior is quite general for bilayers both in model systems and in biological membranes (17). In the present study, liposomes prepared from liver microsomal lipids and labeled with the doxylstearic acids give the ESR spectra shown at the right in Fig. 3. Nearly identical spectra were also obtained with the doxylphosphatidylcholine spin labels. The decrease in overall splitting and the narrowing of the lines as the nitroxide (doxyl) group is translated along the fatty acid chain away from the carboxyl end of the molecule are direct results of increased fluidity as the center of the lipid bilayer is approached. These spectral features, with minor variations, are the same as those observed for a variety of liposomes (e.g., egg phosphatidylcholine vesicles). It is clear from Fig. 3 that these fatty acid spin labels report a fluidity gradient in the bilayer. Overall, however, their mobility is in striking contrast to that seen when the same spin labels bind to intact cytochrome  $b_5$  (see left column, Fig. 3). There is no question that the two environments—the protein surface and the lipid bilayers—have very different effects on the motion of the spin labels.

*Lipid-Protein Binding in Liposomes Containing Cytochrome  $b_5$ .* When the detergent-released cytochrome  $b_5$  is reconstituted with microsomal lipids (see *Methods*) and then labeled with 16-doxylstearic acid, a second more fluid component appears in the ESR spectrum. This composite spectrum is reminiscent of the composite spectra obtained with partially lipid-de-



pleted cytochrome oxidase membranes (1). That is, the spectra appear to consist of one component attributable to lipid binding to the protein overlaid with another component characteristic of lipid bilayer. Electron micrographs of the reconstituted system of microsomal lipids and cytochrome  $b_5$  show considerable heterogeneity, so that quantitative conclusions based on spectral subtractions must be approached with caution. However, on the basis of two reconstitution experiments with different phospholipid levels (5.1  $\mu\text{g}$  of P per mg of protein and 9.5  $\mu\text{g}$  of P per mg of protein) the results obtained by spectral titration and integration show substantial agreement. The calculations are similar to those used in characterizing the lipid-binding properties of cytochrome oxidase (18), and consist of calculating the proportion of the absorption contributed by each of the two putative components of the composite spectrum. Such calculations suggest that each mole of detergent-released cytochrome  $b_5$  immobilizes approximately 2–4 moles of microsomal phospholipid. This estimate also assumes that the binding of the fatty acid spin label is similar to the binding of phospholipid molecules. While this is not subject to direct experimental verification in these experiments, it is possible to test whether cytochrome  $b_5$  (detergent-extracted) binds phospholipids as well as fatty acids by using the doxylphospholipid spin labels (IV, V).

When the phospholipid spin labels interact with cytochrome  $b_5$  (detergent-extracted), the spectra also show strong immobilization. In this case, while the outside splittings are similar to those obtained with the fatty acids, the line shape of the 16-doxylphosphatidylcholine bound to intact cytochrome  $b_5$  suggests the possibility that the spectrum contains a second component with slightly less immobilization. This lineshape difference between protein-bound fatty acid spin labels and protein-bound phospholipid spin labels is very similar to that seen when the two classes of probes interact with bovine serum albumin or with depleted cytochrome oxidase (unpublished observations). In each case the high and low field line positions are unchanged, but the line shapes differ somewhat. One obvious interpretation is that only one of the two side chains of each phospholipid molecule is interacting directly with the protein surface. Another less likely possibility is that the lineshape difference reflects binding to a protein site that is different from the fatty acid site. In any case, it is clear that this binding occurs only in the hydrophobic segment of the intact molecule of cytochrome  $b_5$ . Although the suspected composite nature of the spectra obtained with 16-doxylphosphatidylcholine increases the difficulties encountered in spectral analysis, both classes of lipid spin labels (fatty acid and phospholipid) are clearly binding to the protein in such a fashion that molecular motion is severely restricted.

Spin labels I–III were also diffused into membranes of the microsomal fraction. The ESR spectra (not shown) resemble those observed in the liposomes (see Fig. 3). At the high lipid content found in the membranes, lipid binding to protein may be obscured by the signal from the bilayer regions. This phenomenon was observed in membranous cytochrome oxidase (1). In that case, summing the two isolated spectral components clearly showed that a sizable fraction (30–40%) of the total absorption could be contributed by a highly immobilized spin label and not be visually evident except for very slight peak-to-peak line broadening of the spectrum

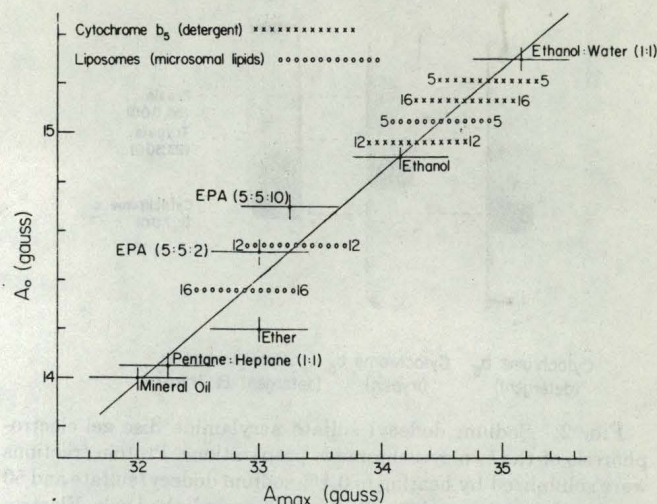


Fig. 4. The solvent dependence of  $A_0$  and  $A_{\max}$ . The values for cytochrome  $b_5$  (detergent) are plotted on the standard curve for homogeneous solvents from Fig. 4 of ref. 20. The lengths of the horizontal and vertical lines indicate the estimated errors in  $A_{\max}$  and  $A_0$ . EPA is a mixture of diethylether:isopentane:ethanol in the ratios indicated. Note that whereas the liposomes (O O O O) show a pronounced polarity gradient, the lipid spin labels bound to cytochrome  $b_5$  (X X X X) all reflect a relatively polar environment.

from the bilayer. With the membranes of the microsomal fraction, it is not possible to demonstrate directly from the experimental data that the immobilized lipid still persists in the membrane. However, the experiments of reconstituting cytochrome  $b_5$  (detergent-extracted) with limited amounts of phospholipid show that lipid-protein binding persists in the presence of contiguous bilayer regions, and suggests that the amount of lipid immobilized by the protein surface remains relatively constant in the presence of adjacent lipid bilayers.

*The Lipid Binding Sites Are More Polar Than the Interior of the Bilayer.* There is a small effect of solvents on the ESR spectra of nitroxide spin labels (19), with the coupling constants being affected by the polarity of the solvent. A semi-quantitative treatment of these solvent effects has been developed and used to estimate the shape of the hydrophobic barrier in lipid bilayers (20). Operationally, relative solvent effects on the coupling constant can be measured either from the sharp three-line spectrum of the spin label tumbling rapidly in solution ( $A_0$ ) or from the two outermost extrema of the spectrum taken in the absence of molecular motion ( $2 A_{\max}$ ). Under ideal conditions  $A_{\max} = A_{zz}$ , where  $A_{zz}$  is the maximum observable anisotropic splitting (corresponding to the magnetic field along the N-O  $2p_z$  orbitals sharing the unpaired electron).  $A_{\max}$  is equal to  $A_{zz}$  only in the absence of molecular motion and interactions between spin labels. The ESR lines of the low temperature spectrum are broad and it is difficult to establish criteria for the absence of these effects, consequently, the estimate of  $A_{zz}$  must be regarded as a crude approximation. With these limitations in mind,  $A_{\max}$  values were determined using spin-labeled cytochrome  $b_5$  (detergent-extracted) and vesicles of microsomal lipids at  $-196^\circ$ . The data are shown in Fig. 4 compared to reference data on  $A_0$  and  $A_{\max}$  of the spin labels in homogeneous solvents (20). (The 5-, 12-, and 16-fatty acid spin labels yield approximately



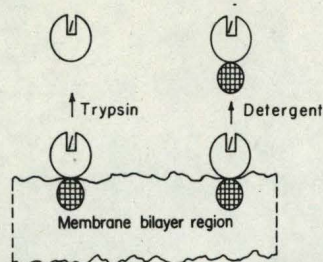


FIG. 5. Highly diagrammatic representation of the relationship between the two cytochromes  $b_5$  (detergent-extracted and trypsin-extracted) and their relationship to the membrane. The cross-hatched regions indicate hydrophobic surfaces buried within the membrane.

the same values for  $A_0$  and  $A_{\max}$  for any given homogeneous solvent of Fig. 4.) There is no accurate way to measure the isotropic parameter,  $A_0$ , because in these preparations the spin labels are not undergoing completely isotropic rapid tumbling, so the protein and lipid data are plotted along the reference line according to the experimental  $A_{\max}$  values.

As can be seen from Fig. 4, there is a distinct polarity gradient across the bilayers of the microsomal lipids. As might be expected, the interior of the bilayer is less polar than near the aqueous interface. This gradient is abolished by dehydration of the samples over phosphorus pentoxide, so that the more polar environment near the interface (as sensed by 5-doxylstearic acid or the corresponding phosphatidylcholine spin label) is largely dependent on the presence of water (20). In contrast, no corresponding polarity gradient is observed in the lipid binding sites of the protein, nor is there any significant change in  $A_{\max}$  for any of the bound spin labels when the protein samples are dehydrated. The lipid spin labels bound to the native cytochrome  $b_5$  all sense an environment with roughly the same polarity.

In addition, we conclude that the lipid binding regions on the hydrophobic segment of the cytochrome  $b_5$  molecule are significantly more polar than the interior of the phospholipid bilayer. This may be due to hydrogen bonding between the polypeptide and the N-O moiety of the spin label. The protein and lipid environments are both hydrophobic in the usual sense, but they are clearly not equivalent. We have found similar results in binding the fatty acid spin labels to lipid-depleted cytochrome oxidase (unpublished observations) and this is evidently a general characteristic of the lipid binding regions of proteins.

**Conclusions.** The intact cytochrome  $b_5$  molecule has a hydrophobic lipid binding surface confined to only one region of the protein, the single peptide segment not present in the trypsin-released portion of the molecule. This tends to confirm the idea (3, 4) that this hydrophobic tail is responsible for anchoring the molecule in the lipid bilayers of the membrane as shown diagrammatically in Fig. 5. Experiments on re-binding cytochrome  $b_5$  with microsomes have demonstrated that the intact cytochrome  $b_5$  can effectively interact with the membranes, and the cytochrome  $b_5$  segment released by hydrolytic means does not interact with the membranes (4). This is consistent with the conclusions from the present spin labeling data, i.e., that lipid binding surfaces are unique to

the intact cytochrome  $b_5$  molecule and are not present in the heme-containing segment. The lipid on the surface of this hydrophobic tail is strongly immobilized (but with an undetermined binding constant), in striking contrast to lipid mobility in the bilayer regions of the membrane. This behavior is very similar to the immobilized layer of lipid (boundary lipid) surrounding the mitochondrial cytochrome oxidase complex (1). The hydrophobic surface of the protein not only immobilizes the lipid it binds, but it can be characterized as somewhat more polar than the interior of the lipid bilayer. In the membrane, native cytochrome  $b_5$  evidently exists as a complex of lipid and protein submerged in the bilayer, with the hydrophilic heme-containing segment extending into the cytoplasm.

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